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Immunization with Live Recombinant *Salmonella typhimurium aroA* Producing F1 Antigen Protects against Plague

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An attenuated *Salmonella typhimurium* strain which expressed the F1 capsular antigen of *Yersinia pestis* was constructed by transformation of *S. typhimurium* SL3261 with plasmid pFGAL2a, a derivative of pUC18 which contained the *caf1* gene without the leader sequence. The recombinant was used to vaccinate mice intragastrically and intravenously. The immunity induced was able to protect mice against challenge with a virulent strain of plague. Protection correlated with the induction of high titers of immunoglobulin G in serum samples and a specific T-cell response.

Yersinia pestis is the causative organism of plague in a wide range of animals and in humans. It is extremely virulent and results in a high rate of mortality. Its virulence is due to a complex array of factors encoded by both the chromosome and three plasmids. These virulence factors include the Lcr genes (33), a fibrinolysin (32), and a capsule.

The vaccines currently available for the prevention of plague are either killed or attenuated whole-cell preparations. They are highly heterogeneous with variable endotoxin content and cause a range of side effects, making them unsuitable for widespread use (17, 22, 26). Moreover, such vaccines are suitable only for parenteral delivery, and although they may effectively induce systemic immunity, parenteral immunization may not be effective as a means of inducing immunity at vulnerable mucosal surfaces (20). The identification of protective subunits of *Y. pestis* which could be delivered directly to mucosal surfaces to induce protective responses may facilitate the development of an improved vaccine against plague. One such candidate subunit is the fraction 1 (F1) antigen, which is a major component in heterogeneous whole-cell vaccines (9, 36).

The capsule that surrounds *Y. pestis* cells is composed of a protein-polysaccharide complex; the purified protein component is F1 antigen (2), with a molecular mass of 17.5 kDa (3). F1 is fully expressed only at 37°C and is believed to confer resistance to phagocytosis, possibly by forming aqueous pores in the membranes of phagocytic cells (27). The detection of antibodies against F1 is the basis of standard serological tests for the surveillance and diagnosis of plague as infected animals and patients produce a strong antibody response to this antigen (30, 36).

The *caf1* gene that encodes F1 antigen has been cloned and sequenced previously (12). Intramuscular (i.m.) immunization of BALB/c mice with recombinant F1 purified from *Escherichia coli* induced high titers of F1-specific antibody and conferred protection against parenteral challenge with 10^5 virulent plague bacilli (31). Other workers have shown that F1 is immunogenic in humans by the subcutaneous route and that the human antiserum so derived affords significant passive protection in mice (23). Purified F1 has been shown not to induce protective immunity by the intragastric (i.g.) route, although it did induce protective immunity by intraperitoneal (i.p.) administration in mice (35). The lack of response following oral immunization may have been due to either inadequate dosing with F1, resulting in the induction of oral tolerance, or destruction of the antigen by stomach acid or digestion.

Attenuated aromatic acid-dependent (*aro*) *Salmonella typhimurium* strains have been well characterized as carriers for various heterologous antigens (7, 10). These vaccine strains are capable of colonization of the gut mucosa and can therefore be used to deliver foreign antigens directly to gut-associated lymphoid tissue, such as Peyer's patches, effectively inducing a local mucosal immune response in the gut. Immune stimulation of the gut mucosa allows induction of immunity via the common mucosal immune system (4, 19) at other distant mucosal sites, particularly the respiratory tract (1, 11). In mice, *S. typhimurium* organisms delivered orally can also achieve systemic invasion from the mucosal surface of the gut to the lamina propria and then via draining mesenteric lymph nodes to the liver and spleen (8, 15, 18, 25) with the subsequent induction of systemic immunity.

In this study, the cloned *caf1* gene was expressed in an *aroA S. typhimurium* mutant and the resulting construct was assessed for the ability to protect mice by the i.g. route against challenge with a virulent plague strain.

MATERIALS AND METHODS

Bacterial strains and cultivation. *Y. pestis* MP6 was grown aerobically at 28°C in blood agar base broth (pH 6.8) which contained the following (in grams per liter of distilled water): Proteose Peptone, 15; liver digest, 2.5; yeast extract, 5; NaCl, 5; supplemented with 80 ml of 0.25% hemin in 1/100 N NaOH. *S. typhimurium* SL3261 (13) and LB5010 (18) were kindly provided by B. A. D. Stocker, Stamford University, and G. Dougan, Imperial College, London, United Kingdom, respectively. *E. coli* JM109 and *S. typhimurium* strains were cultured and stored as described by Sambrook et al. (29).

Cloning of *caf1*. DNA was isolated from *Y. pestis* by the method of Marmur (16). A DNA fragment that encoded the open reading frame of F1 without its signal sequence was amplified by PCR. Oligonucleotides were prepared with a Beckman 200A DNA synthesizer for use in PCR. Oligonucleotide GATC **GAGCTC**GGCAGATTTAACTGCAAGCACC (F1/5′B) corresponds to the first 21 bases of *caf1* immediately following the nucleotide sequence which encodes the F1 signal sequence (underlined) with an additional 5′ region which encodes a *Sac*I site (boldface). Oligonucleotide CAGGTC**GAGCTCGTCGAC**G GTTAGGCTCAAAGTAG (F1/3′A) is complementary to the sequence which encodes a putative stem-loop structure downstream of the *caf1* termination codon, to which a 5′ region which encodes *Sac*I and *Acc*I sites (boldface) has been added. A DNA fragment was obtained after 35 cycles of amplification (95°C, 15 s; 50°C, 15 s; 72°C, 30 s) (Perkin-Elmer 9600 GeneAmp PCR system). The fragment was purified, digested with *Sac*I and *Acc*I, and cloned into plasmid pUC18 to form an in-frame fusion with the initial 17 amino acids of β-ga-

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lactosidase, expressed from the *lac* promoter. This construct was designated pFGAL2a and transformed into *E. coli* JM109 by electroporation. This plasmid construct was designated pFGAL2a. After transformation into *E. coli* JM109, the plasmid was isolated, its authenticity was confirmed, and the plasmid was electroporated into *S. typhimurium* LB5010. Plasmid pFGAL2a isolated from *S. typhimurium* LB5010 was electroporated into *S. typhimurium* SL3261. This construct was designated *S. typhimurium*/pFGAL2a. A culture of *E. coli* which contained the pFGAL2a plasmid was grown at 37°C with shaking in Luria broth which contained 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 18 h. Whole-cell lysates and periplasmic and cytoplasmic fractions of bacteria were prepared as described by Sambrook et al. (29).

Expression of F1 in *S. typhimurium*. The pFGAL2a plasmid was isolated by the method described by Sambrook et al. (29). Purified plasmid was electroporated into *S. typhimurium* LB5010 (r^-m^+). Modified pFGAL2a was subsequently isolated from LB5010 for electroporation into *S. typhimurium* SL3261 (*aroA his*).

The expression of F1 was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). These procedures were performed as described by Hunter et al. (14). Purified F1 (2 mg ml^{-1}) was produced and purified from bacterial cell culture (3). Blots were probed with F1-specific polyclonal antisera raised in sheep immunized with Cutter USP vaccine (dose equivalent to 4×10^9 formaldehyde-killed *Y. pestis* organisms), and bound antibody was detected with a horseradish peroxidase-labelled donkey anti-sheep immunoglobulin G (IgG) antibody (Sigma). Alternatively, IgA monoclonal antibody (MAb) F13G8-1 (American Type Culture Collection) or IgG MAb YPF1-II (U.S. Army Medical Research Institute for Infectious Diseases), both specific for native F1, was used in Western blots. Periplasmic and cytoplasmic fractions were prepared for SDS-PAGE and Western blotting.

S. typhimurium constructs were checked for auxotrophic phenotype by the inability to grow on minimal media which contained histidine without supplements of aromatic amino acids and for the expression of smooth lipopolysaccharide by lysis with phage P22 before immunization experiments.

Quantification of F1 production during in vitro culture. *S. typhimurium*/pFGAL2a and *S. typhimurium*/pUC18 constructs were grown in Luria broth which contained 25 μg of ampicillin ml^{-1}. The viable cells in each culture were enumerated on L-agar which contained 25 μg of ampicillin ml^{-1}. Aliquots of cells removed from culture in stationary or logarithmic phase were treated as follows: cells were harvested by centrifugation and resuspended in 1 ml of phosphate-buffered saline (PBS). The cell suspension was frozen (−20°C), thawed, and sonicated 12 times for 30 s each time (Braun sonicator at maximum power; 25-mm-diameter probe) on ice. Sonicates were analyzed for F1 content by enzyme-linked immunosorbent assay (ELISA).

Each sample and a standard solution of F1 (10 μg/ml) were doubly diluted in PBS across a microtiter plate. After incubation overnight at 4°C, well contents were removed and replaced with 100 μl of blocking solution (1% skim milk powder in PBS) per well. After blocking for 1 h at 37°C, the plate was washed three times in PBS with 0.02% Tween, and 100 μl of mouse polyclonal anti-F1 serum diluted 1:500 in blocking solution was aliquoted into each well and incubated for 1 h at 37°C. The plate was washed, and 100 μl of peroxidase-labelled anti-mouse IgG (Sigma) diluted 1:1,000 in blocking solution was aliquoted into each well. After incubation (1 h, 37°C), the plate was washed five times and 100 μl of 2,2′-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (ABTS) (Sigma) was added to each well. The A_{414} of the plate was read. From absorbance readings, a standard curve was constructed and the concentrations of F1 in samples were derived.

Stability of constructs. For inoculation into mice, bacteria were grown statically at 37°C overnight in Luria broth which contained 25 μg of ampicillin ml^{-1}. Bacteria were washed and diluted in PBS as required. Five female BALB/c mice (supplied and used at 6 weeks of age; Charles River Laboratories) were inoculated intravenously (i.v.) with either 5×10^5 or 5×10^7 *S. typhimurium*/pFGAL2a constructs in 200 μl of PBS. Control mice were inoculated similarly with *S. typhimurium*/pUC18 constructs. Mice showed no ill effects following inoculation. After 7 days, mice were sacrificed by cervical dislocation and livers and spleens were removed. Organs were homogenized in 10 ml of PBS with a stomacher (Seward Medical Ltd.) on maximum setting for 2 min. The homogenate was serially diluted in PBS and plated onto L-agar or L-agar which contained 25 μg of ampicillin ml^{-1}.

Immunization of mice. Eighteen male BALB/c mice (supplied and used at 6 weeks of age; Charles-River Laboratories) were inoculated i.v. on days 1, 7, and 14 with 5×10^7 *S. typhimurium*/pFGAL2a constructs in 200 μl of PBS. An equal number of age-matched mice ($n = 18$) were dosed i.g. by gavage with approximately 10^{10} *S. typhimurium*/pFGAL2a constructs in 200 μl of PBS on days 1, 7, and 21. Ampicillin trihydrate suspension (150 mg ml^{-1}; Penbritin injectable suspension POM; SmithKline Beecham Animal Health) was given subcutaneously for 5 days (50 μl daily) after each inoculation in the hope of stabilizing the plasmid. Control mice were immunized i.v. ($n = 18$) and i.g. ($n = 18$) with *S. typhimurium*/pUC18, i.m. ($n = 15$) with Cutter USP vaccine (dose equivalent to 4×10^8 organisms), or i.p. ($n = 15$) with 10 μg of purified F1 in 200 μl of a 1:1 emulsion of PBS with incomplete Freund's adjuvant (Sigma).

Challenge of immunized mice. Six weeks after the final immunizing dose, five mice from each i.g. and i.v. inoculated and control group were anesthetized i.p. with a cocktail of Domitor (Norden Laboratories) (6 mg $dose^{-1}$) and Ketalar (Parke-Davis) (27 μg $dose^{-1}$), blood was sampled by cardiac puncture, and then the mice were sacrificed by cervical dislocation with removal of livers and spleens. Livers were homogenized and plated onto L-agar to confirm that all *Salmonella* organisms had been cleared. The 10 remaining mice in each group were challenged subcutaneously with a mean dose of 51 CFU of *Y. pestis* GB per mouse. Strain GB was isolated from a plague patient and has been shown to be virulent in mice with a 50% lethal dose (LD_{50}) of approximately 1 CFU per mouse (28).

Analysis of samples. (i) Blood serum. Serum samples were screened for antibodies (IgG and IgA) specific for F1 by using a modification of the ELISA procedure described above. Samples were aliquoted into microtiter wells coated with a constant amount of F1 in PBS (2 μg ml^{-1}) and serially diluted on plates in PBS which contained 1% skim milk powder. After incubation for 1 h at 37°C, samples were removed and the plate was washed before being probed with a peroxidase-labelled secondary antibody (Sigma) directed against either mouse IgG or mouse IgA. Peroxidase-labelled conjugates were each used at a dilution of 1:1,000. The amount of peroxidase-labelled conjugate bound to the plate was determined by the addition of ABTS. Serum samples from individual animals were screened to determine the titer range; samples were subsequently pooled by treatment group to determine the mean titer. The titer was taken as the $\log_{10}$ of the final sample dilution that gave an optical density of >0.1 after subtraction of the nonspecific binding provided by pooled serum samples from age-matched untreated control animals.

(ii) Spleen cells. A crude suspension of mixed spleen cells was prepared by gently grinding each spleen on a fine wire mesh to tease out cells. Cells were flushed from the splenic capsule and connective tissue with 1 ml of tissue culture medium (Dulbecco minimal essential medium with 4% 20 mM L-glutamine, 10^5 U of penicillin $liter^{-1}$, and 100 mg of streptomycin $liter^{-1}$). The cell suspension was gently washed by centrifugation (10 min, 500 rpm, Jovan), and the cell pellet was resuspended in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum. A mixed acridine orange (0.0003% [wt/vol])-ethidium bromide (0.001% [wt/vol]) stain was used to determine the percentage of viable cells in the preparation.

Assay of spleen cell proliferation in vitro against F1 antigen. Doubling dilutions of F1 antigen in the range of 40 to 0 μg ml^{-1} were made in the wells of a microtiter plate so that 100 μl per well remained. Similarly, doubling dilutions of a whole-cell lysate of *S. typhimurium* were made in the range of 50 to 0 μg of protein ml^{-1}. The mitogen concanavalin A (Sigma) was included in the assay in the range of 50 to 0 μg ml^{-1} as a positive control. Equivalent volumes of medium alone provided the negative controls.

The mixed spleen cell suspension was seeded in 100-μl aliquots into each well at a minimum cell density of 5×10^5 cells per well (final volume, 200 μl per well), and incubation was continued for 72 h (37°C; 5% CO_2). Four to 6 h before the end of incubation, [^{3}H]thymidine (1 μCi) was aliquoted to each well in 10-μl volumes (methyl-[^{3}H]thymidine S.A. [74 GBq $mmol^{-1}$]; Amersham). After 72 h, the plate was harvested onto glass fiber filters (Titertek cell harvester), and discs which represented each microtiter well were punched from filter mats into 1.5 ml of scintillation fluid (Cytoscint; ICN Biomedical Inc.) for the measurement of incorporated [^{3}H]thymidine into cells. The stimulation index is derived as follows: mean counts per minute of treated cells/mean counts per minute of controls (four replicates of each).

RESULTS

Cloning and expression of *caf1*. Plasmid pFGAL2a was stably maintained in the *aroA* strain *S. typhimurium* SL3261 following repeated subculture in nonselective broth media. In Western blots of both *E. coli* and *S. typhimurium* SL3261/pFGAL2a, a band (M_r, ~19,000) was recognized by both polyclonal and monoclonal (IgA) anti-F1 reagents (Fig. 1). However, this band was not recognized in blots probed with the anti-F1 IgG MAb. No equivalent band was observed for bacteria which contained only plasmid pUC18. F1 antigen was expressed only by *E. coli* JM109 in the presence of the inducer IPTG; however, expression in *S. typhimurium* SL3261 was constitutive as the *lacI* gene which encodes a repressor is absent in this strain. In Western blots of periplasmic and cytoplasmic *Salmonella* cell fractions, large quantities of F1 were detected in the cytoplasm but not in the periplasm.

Quantification of F1 production in vitro. The production of F1 antigen by *S. typhimurium* SL3261 in vitro was examined. In logarithmic growth phase, an *S. typhimurium*/pFGAL2a culture with a viable count of 1.27×10^8 CFU ml^{-1} yielded 15.92 ± 4.1 μg of F1 ml^{-1}, whereas in stationary phase after overnight culture, 2.36 ± 0.12 mg of F1 ml^{-1} was produced by 4.9×10^8 CFU ml^{-1}. F1 was not detected by ELISA from 1.83×10^8

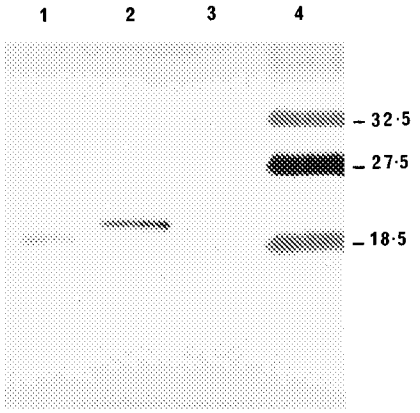

FIG. 1. Analysis of F1 antigen and recombinant F1 antigen by SDS-PAGE and Western blotting. Samples of purified F1 antigen (lane 1) or whole-cell lysates of *S. typhimurium* which contained pFGAL2a (lane 2) or pUC18 (lane 3) were separated by SDS-PAGE, Western blotted, and reacted with an anti-F1 MAb. Prestained molecular size markers (in kilodaltons) were run in lane 4.

CFU of *S. typhimurium*/pUC18 ml^{-1} in log phase or from 1.15×10^9 CFU ml^{-1} in stationary phase after overnight culture.

Stability of *S. typhimurium*/pFGAL2a in vivo. Although the construct was stable in vitro, it was necessary to determine whether *S. typhimurium*/pFGAL2a was also stable in vivo and whether the colonizing ability of *S. typhimurium* was impaired by possession of the plasmid.

After inoculation with one dose of 5×10^5 *S. typhimurium* SL3261 organisms which contained either pUC18 or pFGAL2a, colonization levels in mice were low. A mean of 7.2×10^2 bacteria was isolated from spleen tissue, and a mean of 1.1×10^3 bacteria was isolated from liver tissue. Of these, only 5% of bacteria in spleens and none of the bacteria which had colonized livers retained plasmid pFGAL2a and were able to grow in the presence of ampicillin. A higher level of colonization was achieved following inoculation with 5×10^7 bacteria (Table 1). Similar levels of colonization were observed with *S. typhimurium* organisms which possessed pUC18 or pFGAL2a. A large proportion (85%) of bacteria which colonized spleens still possessed the pUC18 plasmid. However, the insertion of the *caf1* gene into the plasmid resulted in the plasmid becoming unstable in vivo; only 0.52 to 0.88% of colonizing *Salmonella* organisms were able to grow in the presence of ampicillin. Therefore, mice immunized subsequently with *S. typhimurium*/pFGAL2a were dosed with ampicillin for 5 days following each inoculation in the hope of stabilizing the plasmid in vivo.

Antibody titer to F1 in immunized groups. The induction of specific antibodies in mice inoculated either i.g. or i.v. with *S. typhimurium*/pFGAL2a was examined. This was compared with the antibody response induced by the formaldehyde-killed Cutter USP vaccine.

TABLE 1. Stability of plasmids in vivo[a]

Plasmid	Organ	CFU/organ enumerated on:		% Retaining plasmid
		L-agar	L-agar + ampicillin	
pUC18	Liver	1.2×10^5	1.6×10^4	13
	Spleen	4.8×10^4	4.1×10^4	85
pFGAL2a	Liver	8.4×10^4	4.4×10^2	0.52
	Spleen	7.9×10^4	7.0×10^2	0.88

[a] Five BALB/c mice were inoculated i.v. with 5×10^7 *S. typhimurium* organisms which contained pFGAL2a or pUC18. Livers and spleens were removed 7 days later and homogenized in PBS before being plated on L-agar or L-agar which contained 25 μg of ampicillin ml^{-1}.

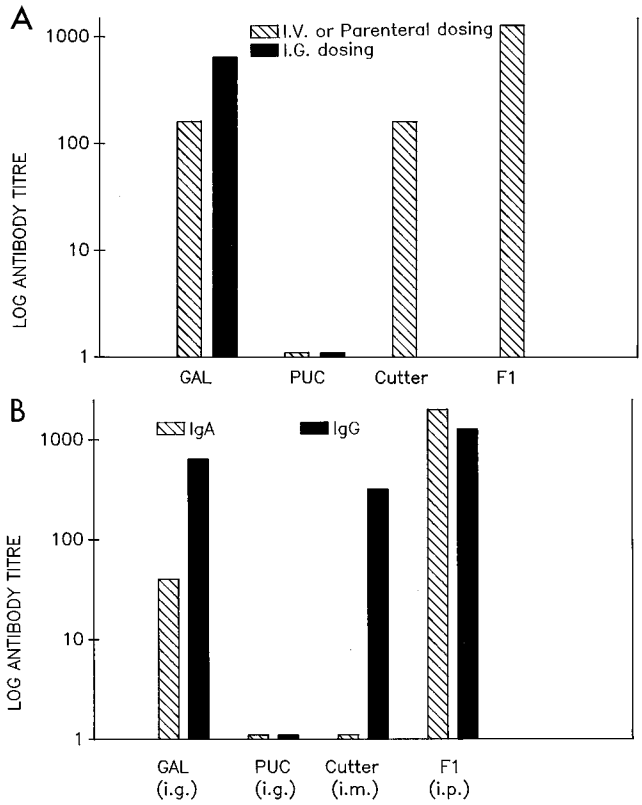

FIG. 2. F1-specific titers of IgG (A) and of IgG and IgA (B) in pooled serum samples from immunized animals 6 weeks after dosing. GAL, *S. typhimurium*/pFGAL2a; PUC, *S. typhimurium*/pUC18.

The titers of F1-specific IgG in pooled serum samples from immunized animals 6 weeks after either i.g. or i.v. dosing with *S. typhimurium*/pFGAL2a are shown in Fig. 2A. These titers are compared with the titers in serum induced by parenteral immunization with the Cutter USP vaccine and purified F1. It can be seen that *S. typhimurium*/pFGAL2a administered i.v. was as effective as the Cutter USP vaccine in inducing an F1-specific IgG titer of 1:160. Dosing with *S. typhimurium*/pFGAL2a i.g. induced a higher titer of IgG specific for F1 (1:640), indicating that bacteria had achieved systemic invasion from the gut mucosa and had induced systemic immunity. Thus, i.g. dosing with this *Salmonella* construct can induce and sustain titers of IgG in serum samples equivalent to those which result from i.v. dosing.

A comparison of F1-specific IgG and IgA titers in serum samples 6 weeks after i.g. dosing with *S. typhimurium*/pFGAL2a (Fig. 2B) demonstrated that IgA titers were approximately 16-fold lower than IgG titers (1:40 [compared with 1:640]). i.v. dosing with *S. typhimurium*/pFGAL2a did not result in significant titers of IgA in serum samples after 6 weeks (results not shown), nor did i.m. dosing with the Cutter USP vaccine. However, i.p. dosing with F1 alone induced the highest titers of specific antibody (IgA and IgG) in serum samples (1:2,000 and 1:1,280, respectively), and this may be attributed to the relatively high immunizing dose (10 μg) and to the fact that of all the parenteral routes, the i.p. route is the only one known to induce mucosal immunity.

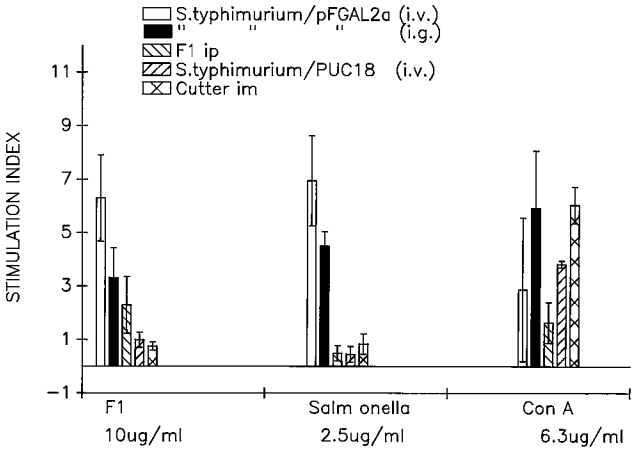

FIG. 3. Spleen cell proliferation against F1 antigen, *Salmonella* lysate, and concanavalin A (Con A). Five mice from each treatment group were humanely killed on day 63 of the immunization schedule with the removal of spleens. Spleen cells were pooled by treatment group and cultured in vitro for 72 h, and proliferation was estimated by the incorporation of [^{3}H]thymidine into DNA. The stimulation index is derived as follows: mean counts per minute of treated cells/mean counts per minute of controls ± the standard error of the mean (four microtiter wells).

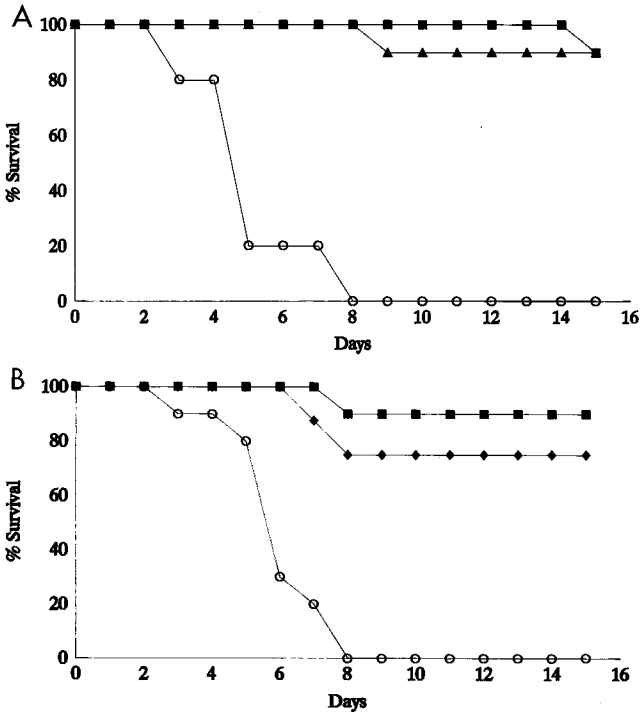

FIG. 4. Survival of BALB/c mice following challenge with a virulent plague strain. Mice immunized i.g. (A) or i.v. (B) with *S. typhimurium* organisms which contained pFGAL2a (■) or pUC18 (○), i.m. with Cutter USP vaccine (▲), or i.p. with purified F1 antigen (◆) were challenged with 50 LD_{50}s of *Y. pestis* GB.

Within treatment groups, individuals did not show a great variation in antibody titer. For example, at a sample dilution of 1:80, the mean optical density at 414 nm ± the standard error of the mean from titration of IgG for individual serum samples was 0.190 ± 0.003 for the pFGAL2a i.v. group, 0.209 ± 0.015 for the Cutter i.m. group, and 0.288 ± 0.052 for the F1 i.p. group.

Spleen cell responses in vitro. The ability of the *S. typhimurium*/pFGAL2a construct to induce a cell-mediated immune (CMI) response was determined by assaying the proliferation of spleen cells in vitro on reexposure to purified F1.

Maximum proliferation in response to F1 was observed in spleen cells derived from mice dosed i.v. with *S. typhimurium*/pFGAL2a (Fig. 3). Spleen cells derived from mice dosed with F1 (i.p.) and with *S. typhimurium*/pFGAL2a (i.g.) also proliferated in response to F1, but to a lesser extent. Negligible proliferation in response to F1 was observed in spleen cells derived from mice immunized with the Cutter USP vaccine.

Spleen cells derived from mice immunized either i.g. or i.v. with *S. typhimurium*/pFGAL2a proliferated in response to the *Salmonella* protein extract, further indicating that there was systemic involvement in the immune response after i.g. dosing as well as i.v. dosing. The nonspecific proliferative response to the T-cell mitogen concanavalin A demonstrated that spleen cells derived from all sources had equal potential for proliferating.

Protection of immunized mice against plague bacterium challenge. To determine whether immunization with *S. typhimurium*/pFGAL2a provided protection against plague infection, immunized animals were challenged subcutaneously with 50 LD_{50}s of a virulent strain of *Y. pestis*.

Following challenge with *Y. pestis* GB, almost complete protection was obtained with each vaccine used, but all of the negative control mice inoculated with *S. typhimurium*/pUC18 died within 9 days (Fig. 4). One of 10 mice immunized with the Cutter vaccine died, as did 2 of 8 mice immunized i.p. with purified F1, although their survival was prolonged over that of the majority of negative controls. *S. typhimurium* organisms which expressed F1 induced good protection by both i.v. and i.g. routes of inoculation, with no observable deterioration in the health of survivors of this challenge.

DISCUSSION

In this study, we have demonstrated that the cloned *caf1* gene can be expressed in the *aroA* strain *S. typhimurium* SL3261 and that protective immunity was induced in mice by this recombinant organism. The construction of plasmid pFGAL2a, which carries the *caf1* gene that encodes the mature F1 protein but not its signal sequence, allowed the expression of F1 antigen in both *E. coli* and *S. typhimurium* organisms. Recombinant F1 was recognized in Western blots by polyclonal antiserum and an IgA MAb, indicating that the recombinant antigen has retained at least some of the antigenic properties of native F1. The recombinant protein (M_r, ~19,000) was larger than purified F1, reflecting the fact that F1 was expressed as a fusion with part of the β-galactosidase protein. Some antigenic differences might be expected because of different glycosylation states of the molecule expressed in *Y. pestis* and *S. typhimurium* organisms. Evidence for this was provided by the failure of the IgG MAb to recognize recombinant F1 in Western blots.

Although plasmid pFGAL2a was maintained stably in the absence of antibiotic-selective pressure in vitro, in vivo there was a significant reduction in the proportion of bacteria which possessed the plasmid after 7 days. The instability of a number of plasmids which direct the expression of recombinant antigens in *Salmonella* organisms has been observed previously (18). As with this construct, the unstable plasmids employed strong unregulated promoters and high copy numbers. Several approaches for overcoming this problem, including the use of low-copy-number plasmids, balanced lethal mutations, and

chromosomal integration (10), which bypass the need for antibiotics and allow the removal of the antibiotic resistance marker, exist.

Previous work (31) has indicated that the immunizing dose of F1 is critical for protection against plague. In addition, repeated dosing with protein antigens at very low or excessively high levels can induce tolerance. Immune tolerance has been observed previously in mice immunized i.g. with low-level doses of F1 (35). Thus, in designing a *Salmonella*-based F1 vaccine, a substantial level of expression of F1 appears to be desirable. The inoculating dose of 5×10^7 CFU of *S. typhimurium*/pFGAL2a cultured in vitro would have contained 6.3 μg of F1, but it is difficult to estimate the subsequent level of F1 that this inoculum would have produced in vivo. However, the protection observed in mice vaccinated with this construct indicates that the expression levels of cloned F1 were sufficient to induce protective immunity but avoided the low- or high-zone tolerance brackets for i.g. dosing.

The presentation of F1 in a live, attenuated *S. typhimurium* strain has several advantages over the killed vaccine currently available and previously used attenuated *Y. pestis* vaccine strains, such as Haffkine and EV76. In studies with live EV76 (21), excessive adverse reactions were observed and the degree of protection it afforded was highly variable between individuals. In addition, strain EV76 was shown to be lethal for nonhuman primates and did not elicit a measurable circulating antibody response that was significantly better than that induced by the killed vaccine. The killed vaccine has also been shown to cause a range of side effects in humans (17, 22, 26), and pneumonic plague has been reported in vaccinated individuals, indicating that it affords incomplete protection (21).

The *S. typhimurium*/pFGAL2a construct induced a specific anti-F1 response in mice. Six weeks after the last immunizing dose, the titers of F1-specific IgG in samples from animals dosed i.g. or i.v. with *S. typhimurium*/pFGAL2a were measurable. The mean titer of IgG in serum samples from mice dosed i.v. with recombinant *S. typhimurium* was equivalent to that induced by the Cutter USP vaccine. i.g. dosing with the *S. typhimurium*/pFGAL2a construct was as effective as i.v. dosing at inducing seroconversion.

The induction of a mucosal secretory IgA response may be a requirement for an improved plague vaccine to protect against the highly contagious pneumonic form of plague. *Salmonella* organisms invade the intestinal mucosa by direct interaction with the follicular lymphoid tissues of the gut. It has been shown that the multiplication of bacteria in gut-associated lymphoid tissue leads to a secretory IgA response (24). *Salmonella* organisms can also induce a CMI response as they are able to multiply intracellularly (5). In animals immunized i.g. with *S. typhimurium*/pFGAL2a, F1-specific IgA was detected in addition to IgG. This IgA titer in serum samples probably reflects the level of secretory IgA production in the gut after oral dosing, although the assay of IgA in serum samples is only an indirect measurement of secretory IgA production. These data provide preliminary evidence that in addition to inducing seroconversion, i.g. dosing with *S. typhimurium*/pFGAL2a effectively induces mucosal immunity.

The Cutter-immunized group did not have detectable levels of IgA in serum samples; this was expected since i.m. dosing does not effectively stimulate mucosal immunity. The existing Cutter USP vaccine is deficient in this respect. The fact that protection was observed in Cutter-vaccinated mice in this study can be attributed to the induction of systemic immunity protective against the parenteral challenge used.

The induction of a CMI response is a requirement for any ideal plague vaccine to be totally effective. A CMI response must be induced to eliminate bacilli from intracellular sites, as *Y. pestis* is able to survive in intracellular locations, such as within macrophages and phagolysosomes (6, 34). Spleen cells derived from groups immunized with *S. typhimurium*/pFGAL2a either i.g. or i.v. proliferated significantly when restimulated in vitro with purified F1 antigen, indicating that the organism had induced a CMI response. The spleen cell responses in *S. typhimurium*/pFGAL2a groups were greater than those in the Cutter- and F1-immunized groups, indicating that the presentation of F1 in a live-vaccine vector stimulated a CMI response more effectively than did presentation in the killed Cutter USP vaccine or as purified soluble protein. Maximum proliferation against F1 was observed in spleen cells derived from mice dosed i.v. with *S. typhimurium*/pFGAL2a; this illustrates how effectively i.v. dosing induces systemic immunity by recruiting spleen cells into the immune response. In contrast, the i.m. route of immunization required by the Cutter USP vaccine was much less effective at inducing a CMI response. Dosing with the recombinant i.g. also induced a CMI response, indicating systemic invasion of *S. typhimurium*/pFGAL2a organisms from the gut mucosa.

Spleen cells from the *S. typhimurium*/pUC18 group showed little or no response to the *Salmonella* lysate, and we can only speculate on the reasons for this. This spleen cell population showed a normal response to concanavalin A, indicating that cells were functional and had the potential to proliferate in response to other antigens. It may be that in the *S. typhimurium*/pFGAL2a group, *Salmonella* epitopes were presented together with F1 epitopes to T cells, inducing cross-reactive T-cell clones. This would not occur with the empty plasmid construct.

Against parenteral challenge with a virulent plague strain, *S. typhimurium*/pFGAL2a groups exhibited 90% protection, as did the Cutter group. The group immunized with purified F1 showed 75% protection. No protection was seen in the control groups vaccinated with the *S. typhimurium*/pUC18 construct, discounting an earlier suggestion that antigenic determinants common to *Y. pestis* and *S. typhimurium* could afford a degree of nonspecific immunity (31). The possibility of a nonspecific influence in the observed protection against plague was minimized by delaying the challenge for 6 weeks after the last immunizing dose to allow the clearance of *Salmonella* organisms (18).

Thus, we have demonstrated that the F1 antigen of *Y. pestis* is expressed in an attenuated *Salmonella* strain and that it induces 90% protection against challenge by a virulent *Y. pestis* strain. This approach shows potential as the basis for a live attenuated oral vaccine, in which there is increased stable expression of F1 in conjunction with other antigens, to achieve complete protection against *Y. pestis*.

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